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# Evidence for the monomeric nature of thymosins

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According to gel-filtration experiments, α- and β-thymosins appear to form oligomers, which are 4–5-fold larger than the corresponding polypeptides. However, on analysis by sedimentation equilibrium ultracentrifugation, prothymosin α and thymosin $\beta_4$ showed relative molecular masses of 12800 and 4600, which are close to the values calculated from their amino acid sequences, confirming their existence in solution as discrete monomeric entities.

Prothymosin α; Thymosin $\beta_4$; Sedimentation equilibrium; Gel filtration

## 1. INTRODUCTION

Thymosins are peptides of wide distribution in tissues and species (reviews [1,2]), that have been related to cell-mediated immunity phenomena [1,2] and cell proliferation [3,4]. They show similarities in physicochemical properties which could arise from corresponding compositions and partial homologies of sequence [1,2,5]. The $\alpha$- and $\beta$-thymosins are distinguished by their isoelectric points ($\leq 5$ for $\alpha$-, and $\geq 5$ for $\beta$-thymosins) [6]. They are also distinguished by the size of their polypeptides: approx. 100 residues for prothymosin $\alpha$ (110) [3,7] and parathymosin $\alpha$ (101) [8]; or approx. 40 residues: $\beta_4$ (43) [9], $\beta_9$ (41) [10] and $\beta_{10}$ (43) [11].

The molecular weights of $\alpha$- and $\beta$-thymosins were estimated from gel-filtration experiments and found to be several fold greater than those calculated from their amino acid sequences. Therefore, a common feature of these peptides seems to be an apparent tendency to associate to oligomers ranging in size from trimers to hexamers [5]. Recently, thymosin $\alpha_1$ (the 28-residue N-terminal fragment of prothymosin $\alpha$) was found to be monomeric by sedimentation equilibrium ultracentrifugation, indicating the exceptional behaviour of thymosin $\alpha_1$ on gel filtration [5]. In the present paper, evidence is presented on the properties in solution of the major polypeptides of the $\alpha$- and $\beta$-thymosins, namely prothymosin $\alpha$ and thymosin $\beta_4$. The results confirm their anomalous behaviour on gel filtration, and show that they are monomeric according to sedimentation equilibrium distributions following ultracentrifugation.

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## 2. MATERIALS AND METHODS

Prothymosin $\alpha$ was isolated from calf thymus according to the method previously used for rat thymus [12], and thymosin $\beta_4$ was also isolated from calf thymus [10]. Parathymosin $\alpha(1-30)$ was synthesized by the Merrifield solid-phase method [13].

Gel-filtration experiments were carried out according to Haritos et al. [5]. Protein concentrations were determined by specific radioimmunoassays for prothymosin $\alpha$ [14] and parathymosin $\alpha(1-30)$ [15], and by the method of Bradford [16] for thymosin $\beta_4$.

Sedimentation equilibrium experiments were carried out using a Beckman model E analytical ultracentrifuge, fitted with an RTIC unit. The temperature was maintained at 4°C, and the

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initial protein concentration was approx. 7 mg/ml. Before gel filtration and ultracentrifugation experiments the lyophilized peptides were dissolved in phosphate buffer (pH 7.0, $I = 0.1$) + 0.2 M KCl. Values of 0.742, 0.728 and 0.686 ml/g were taken as the partial specific volumes of parathymosin $\alpha(1-30)$, thymosin $\beta_4$ and prothymosin $\alpha$, respectively. These values were calculated [17] from the amino acid compositions. Data on the densities of the salt solutions were taken from International Critical Tables.

Z-average molecular weights were determined from sedimentation equilibrium experiments [18], using Schlieren optics and solution columns of 3 mm. Heterogeneity of preparations was detected by plots of $(1/r \cdot dc/dr)$ vs $(c_r - c_a)$, where $c_r$ and $c_a$ are the concentrations at radial distance, $r$, and at the meniscus, respectively.

## 3. RESULTS AND DISCUSSION

Gel filtration at neutral pH of synthetic parathymosin $\alpha(1-30)$, calf thymosin $\beta_4$ and prothymosin $\alpha$ on Sephacryl S-200 showed apparent molecular masses that are 4–5-fold greater than those calculated from their sequences (table 1, fig.1). These results are similar to the 5.5-fold increase in apparent size on gel filtration of synthetic thymosin $\alpha_1$ [5].

In contrast, sedimentation equilibrium experiments, under the same experimental conditions, indicated that these polypeptides do not associate (table 1, fig.2). The data were limited to z-average molecular weights, obtained directly from the concentration gradients at the end of the period of ultracentrifugation [18]. The linear dependence (fig.2) shows that each of the polypeptides was homogeneous. Furthermore, the relative molecular masses calculated from the slopes gave results that are within 1000 of the expected values (table 1). Although the relative error is high (25%) for the small peptide parathymosin $\alpha(1-30)$, the result does not suggest a multiple size even in this instance. The agreement between observed and expected for thymosin $\beta_4$ and prothymosin $\alpha$ is good, with a relative error of 6–7%. The results confirm that these representatives of the thymosin family are monomeric, as previously shown for thymosin $\alpha_1$ [5].

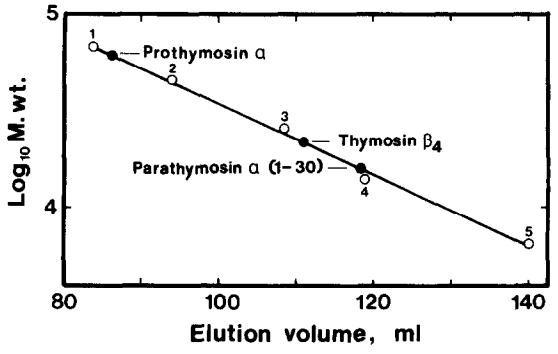


Fig.1. Gel filtration on Sephacryl S-200. The graph shows the elution volumes of calf prothymosin $\alpha$, calf thymosin $\beta_4$, and synthetic parathymosin $\alpha(1-30)$ in relation to the marker proteins: bovine serum albumin (1), ovalbumin (2), chymotrypsinogen A (3), ribonuclease (4) and aprotinin (5). The buffer was phosphate (pH 7.0, $I = 0.1$) + 0.2 M KCl and the temperature was 4°C.

The identity of thymosin $\alpha_1$, i.e. prothymosin $\alpha(1-28)$, with the N-terminal sequence of prothymosin $\alpha$, and its 43% homology with parathymosin $\alpha(1-30)$ could provide a common feature for the similar anomalous behaviour on gel filtration of these three peptides. Thymosin $\beta_4$ also behaves anomalously on gel filtration, and the apparent molecular size is 4-fold greater than expected for the single polypeptide (table 1). Since thymosin $\beta_4$ is similar in size and 78% homologous with thymosin $\beta_9$, and 69% homologous with thymosin $\beta_{10}$, it is likely that all the present

Table 1

Relative molecular masses of synthetic and native thymosins

| Thymosins | $M_{r(calc.)}$ | $M_{r(g.f.)}$ | Ratio[a] | $M_{r(s.e.)}$ | Refs |
|---|---|---|---|---|---|
| Thymosin $\alpha_1$ | 3108 | 17000 | 5.5 | 3200 | [5] |
| Parathymosin $\alpha(1-30)$ | 3345 | 16000 | 4.8 | 4150 | figs 1,2 |
| Thymosin $\beta_4$ | 4963 | 21000 | 4.2 | 4640 | figs 1,2 |
| Prothymosin $\alpha$ | 11983 | 61000 | 5.1 | 12800 | figs 1,2 |

[a] Ratio of relative molecular masses from gel filtration, $M_{r(g.f.)}$ to those calculated, $M_{r(calc.)}$. $M_{r(s.e.)}$ are the relative molecular masses obtained from sedimentation equilibrium experiments

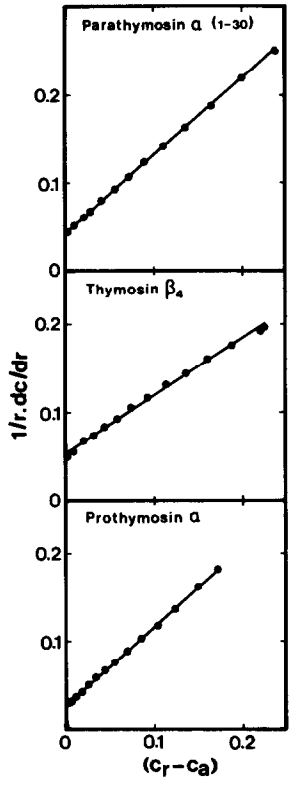


Fig.2. Sedimentation equilibrium giving $M_z$ for parathymosin $\alpha$(1–30), calf thymosin $\beta_4$ and calf prothymosin $\alpha$. The graphs show the ratio of refractive index increment to radial position plotted against the increase in concentration from the meniscus. The slopes correspond to $M_z$ values of 4150, 4640 and 12800 for parathymosin $\alpha$, thymosin $\beta_4$ and prothymosin $\alpha$, respectively. The corresponding rotor speeds and durations were: 42040, 33450 and 21740 rpm and 14, 19 and 19 h for the three polypeptides, respectively. The buffer was phosphate (pH 7.0, $I$ = 0.1) + 0.2 M KCl and the temperature was 4°C.

families of $\alpha$- and $\beta$-thymosins share the same anomalous behaviour on gel filtration.

Since the present sedimentation equilibrium results preclude the aggregation of thymosins, the early elution on gel filtration could be explained by asymmetry or charge repulsion of solute by the matrix. Previous results gave a value of 221 $\mu m^2/s$ for the diffusion coefficient ($D_{20,w}$) of thymosin $\alpha_1$, corresponding to a frictional ratio of 1.0 and indicating a spherical molecule [5]. However, recalculation of that data gives a corrected value of 171 $\mu m^2/s$ for the diffusion coefficient of thymosin $\alpha_1$ and a frictional ratio of 1.3. Thus, asymmetry does make some contribution to the decreased elution volume on gel filtration, but does not completely account for this effect. Therefore, a large part of the phenomenon presumably derives from the charge repulsion by the matrix. This inference is in agreement with the increase in elution volume, and corresponding decrease in apparent molecular size, when gel filtration was carried out at an acidic pH. For example, the apparent size enhancement for prothymosin $\alpha$ drops from being nearly 5-fold at pH 7 to 3-fold at pH 2.8 [5]. The fact that the charge repulsion is not completely lost at low pH reflects the residual high density of negative charges still present owing to the peculiar composition of these acidic proteins [5].

The sedimentation equilibrium experiments were carried out at the high concentration of approx. 7 mg/ml. This value is much higher than those measured in vivo, for example in rat tissues, of between 200 and 400 $\mu$g/g [19,20]. These results tend to the conclusion that thymosins do not form oligomers in vivo, and may exert their biological functions as monomeric entities.

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